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Hydrophobic Interaction Chromatography

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Introduction

According to J.N. Israelachvili (1985), hydrophobic interactions constitute 'the unusually strong attraction between nonpolar molecules and surfaces in water'. For two contacting methane molecules, the attraction energy is about sixfold higher in water than the van der Waals interaction energy in a vacuum. This energy, which has been estimated to be about -8.5 kJ mol^{-1} for two methane molecules is due to the extrusion of ordered water on two adjacent hydrophobic surfaces into less-ordered bulk water with a concomitant increase in entropy. This entropy-driven attraction between nonpolar groups in water is the basis for hydrophobic interaction chromatography.

The chromatographic separation of proteins depends on the differential accumulation of molecules at certain sites within a chromatographic system. Two principal types of chromatographic systems employing hydrophobic media have been described: (a) reversed-phase and (b) hydrophobic interaction chromatography. The principle of reversed-phase chromatography is based on a hydrophobic, e.g., silica, support of very high hydrophobicity which is capable of retaining nonpolar liquid phases (stationary liquid phase) when applied as the less polar phase in a solvent system. In this classical system the solutes are absorbed and separated (partitioned) in the apolar stationary liquid phase (i.e., a three-dimensional system) and not on the solid phase. In hydrophobic interaction chromatography the solutes (proteins) are adsorbed and separated on the apolar stationary solid phase (i.e., a two-dimensional system) carrying immobilized hydrophobic groups. Because of the very different scopes and methodological details, reversed-phase chromatography will not be treated here. The same holds for other forms of liquid–liquid partition chromatography. A differentiation will also not be made between classical chromatographic systems and HPLC since, in essence, it is only the bead or particle size which leads to the higher performance (e.g. throughput, resolution) in the latter method.

Discovery and Development of Hydrophobic Interaction Chromatography

The chromatographic purification of proteins on specifically synthesized hydrophobic solid supports was first reported independently by Yon and Shaltiel in 1972. In both cases the hydrophobic matrix consisted of agarose to which aminoalkane derivatives have been coupled by the CNBr method. Yon synthesized mixed hydrophobic-charged gels (aminodecyl-, or *N*-3-carboxypropionyl)aminodecyl-agarose) with an alkyl residue to charge ratio of at least 1 : 1 for the adsorption of lipophilic proteins such as bovine serum albumin or aspartate transcarbamoylase. These proteins were adsorbed at low ionic strength at the isoelectric point and eluted at acidic or alkaline pH by charge repulsion. The surprising result in Shaltiel's experiments was that a very normal hydrophilic enzyme, phosphorylase *b*, could be purified on hydrocarbon-coated agaroses to near homogeneity in one step, implicitly questioning the general doctrine of the time that all hydrophobic amino acids are buried in the interior of proteins. Phosphorylase was adsorbed at low ionic strength on immobilized butyl residues which had no resemblance to the substrates of the enzyme (excluding affinity chromatography) and was eluted by a 'deforming buffer' which imposed a limited conformational change on the enzyme. Taken together with Shaltiel's systematic approach of grading the hydrophobicity of the gels via an immobilized homologous hydrocarbon series, the immediate

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impression was that here was a novel method applicable not only to hydrophobic or lipophilic but also to hydrophilic, possibly to all proteins. The name 'hydrophobic chromatography' coined by Shaltiel therefore soon came to widespread use. Only a few months after Shaltiel's first paper, B.H.J. Hofstee published a series of papers leading to similar results.

As stated above, all of these hydrophobic gels were synthesized by the simple CNBr method. Some criticism however arose that positive charges, introduced by a side reaction into the matrix by the CNBr procedure, were influencing the chromatographic results on hydrocarbon-coated agaroses. A rational approach and solution to this problem proved difficult since the chemical mechanism of the CNBr coupling reaction was conclusively clarified only some time later by M. Wilchek in 1981. Wilchek found that the number of charges introduced into the matrix dependend on the pH of the washing solution and the length of the washing procedure after CNBr activation of the agarose, since intermediate cyanate esters were selectively hydrolysed in alkali in contrast to the imidocarbonates which were hydrolysed in acid. Thus pure charged isourea gels, pure uncharged imidocarbonate/carbamate gels or mixed ionic–hydrophobic gels can be obtained by the CNBr procedure. In a later paper, Shaltiel conclusively showed that under his conditions the influence of charges in his hydrocarbon-coated agaroses had been small. In addition it was shown by various other groups that salts also effectively quenched the charges introduced by the CNBr method.

Fully uncharged hydrophobic gels were therefore synthesized in 1973 by Porath's group who reacted benzyl chloride with agarose at high temperatures. The synthesis of a graded homologous series of hydrocarbon-coated agaroses was however not possible by this method. In addition Porath demonstrated the inverse salt behaviour of proteins adsorbed on such gels. In contrast to ion exchangers, proteins were applied to these gels at *high salt concentrations* and eluted by decreasing the ionic strength (negative salt gradients). Interestingly the protein cytochrome *c* was adsorbed when 1–3 M NaCl was included in the buffer, a salt which in itself had very little salting-out potential. A similar salt behaviour of protein binding on hydrophobic gels synthesized by the CNBr procedure was reported later by Hjerten who demonstrated that Shaltiel-type gels showed similar properties as the Porath-type gels. Hjerten also suggested the term 'hydrophobic interaction chromatography' which is now popularly accepted. In 1974 Hjerten described a novel preparation of uncharged hydrophobic gels of broad potential by coupling alkyl and aryl groups via the glycidyl ether method.

In retrospect, although there is no doubt that fully uncharged hydrophobic gels are, by virtue of displaying a single (pure) type of noncovalent interaction, superior to the CNBr-prepared gels, it appears that all groups involved in the development of hydrophobic (interaction) chromatography observed the binding and fractionation of proteins by predominantly hydrophobic interactions. Both terms, 'hydrophobic chromatography' and 'hydrophobic interaction chromatography' can be used synonymously, the shorter term 'hydrophobic chromatography' being no more a misnomer than 'affinity chromatography'.

Fundamentals of Hydrophobic Interaction Chromatography

The Chain Length Parameter

A general systematic approach to the purification of proteins via hydrophobic interactions was initiated by Shaltiel who introduced the principle of variation of the immobilized alkyl chain length in the form of the homologous series of hydrocarbon-coated agaroses (Seph-C_n, $n = 1$–10). The major conclusion of his experimental result was that an increase of the chain length by $-CH_2-$ units concomitantly increased the strength of protein binding from retardation to reversible binding up to very tight binding ('irreversible' binding). In addition to this variation in binding affinity with the chain length, the gels also changed their specificity towards the adsorbed protein. Thus it was suggested that the properties of hydrophobic agaroses for protein purification could be optimized by variation of the immobilized alkyl chain length.

The Surface Concentration of Parameter

Critical surface concentration of immobilized residues In 1975 we showed that a second parameter is of equal if not greater importance than the alkyl chain length. If, instead of the chain-length, the density (surface concentration) of immobilized alkyl groups is varied, protein adsorption is a sigmoidal function of the surface concentration of immobilized alkyl residues (**Figure 1**) (i.e., surface concentration series). Here also the strength of binding increased from retardation to very tight binding as in the homologous series of Shaltiel. Figure 1 also illustrates the effect of chain elongation in a homologous series which leads to a leftward shift of the sigmoidal curves and to a loss of sigmoidal shape. Another important finding was that a threshold value of the alkyl surface

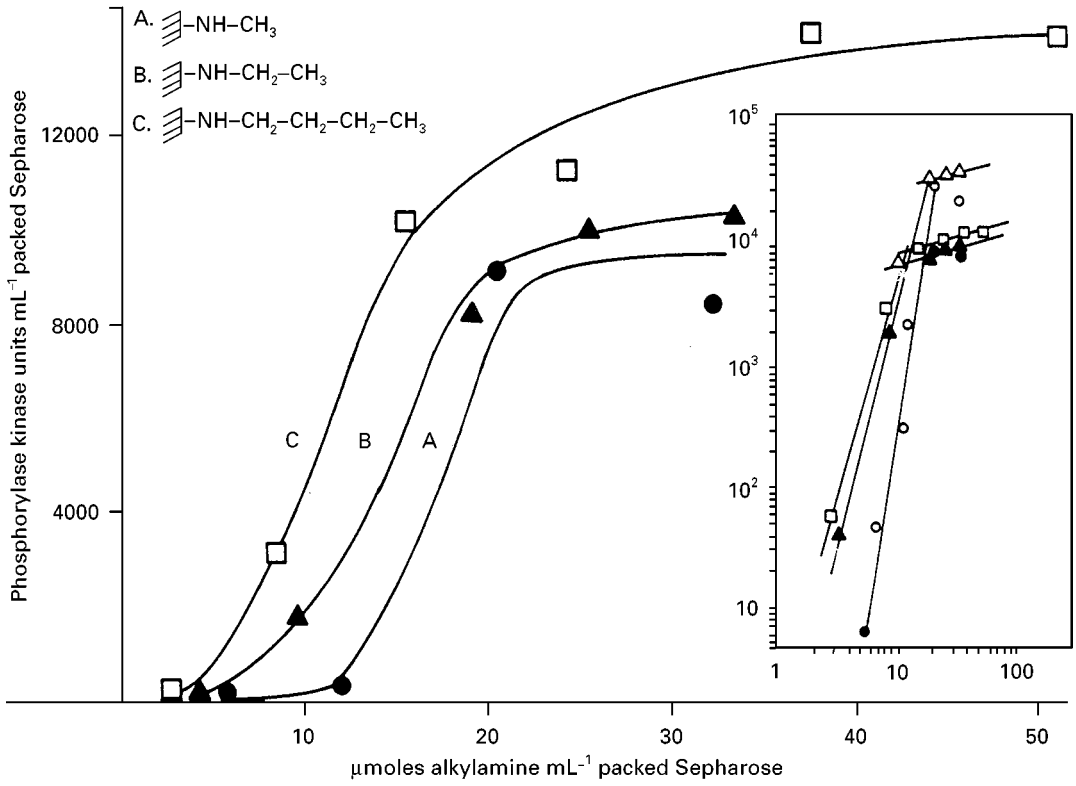

Figure 1 Dependence of the adsorption of phosphorylase kinase on the chain-length and surface concentration parameters of a homologous series of alkyl-Sepharoses at low ionic strength. The amount of adsorbed enzyme activity per mL packed Sepharose was calculated from the difference between the total amount of applied units and the amount excluded from the gel. The crude rabbit muscle extract or purified phosphorylase kinase was applied to columns containing ca. 10 mL packed gel. The alkyl agaroses were synthesized by the CNBr method. The ratio of alkyl residues to positive charges was ca.10 : 1. Inset: Double logarithmic plots of adsorbed phosphorylase kinase as a function of the degree of substitution. Experiments with purified phosphorylase kinase are included. A, Seph-C_1: (●) crude extract; (○) purified phosphorylase kinase. B, Seph-C_2: (▲) crude extract; (△) purified phosphorylase kinase; C, Seph-C_4; (□) crude extract. For further details see the text and Jennissen HP and Heilmeyer Jr LMG (1975) General aspects of hydrophobic chromatography. Adsorption and elution characteristics of some skeletal muscle enzymes. *Biochemistry* 14: 754–760.

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concentration, a 'critical hydrophobicity', had to be reached before a protein adsorbed. With a ratio of alkyl residues to positive charges in the gels of about 10 : 1, the predominance of hydrophobic interactions as the basis for adsorption was strongly indicated. Thus sigmoidal adsorption curves and critical hydrophobicities could also be obtained in the presence of high salt concentrations (see **Figure 2**) excluding the argument that the sigmoidal shape was due to the action of charges. Finally, the same sigmoidal behaviour of protein adsorption was found on uncharged hydrophobic gels at low ionic strength and an example will be shown in this article.

Cooperative interaction of multiple immobilized residues with the protein A straightforward interpretation of the sigmoidal curves (**Figures 1** and **2**) was provided by the concept of multivalence and cooperativity of protein adsorption. It became clear that the sigmoidicity and the 'critical hydrophobicity' were due to the multivalence of the interaction (i.e., the necessity for a simultaneous interaction of more than one alkyl residue with the protein moiety). The term 'multivalence' is to be preferred to other terms such as 'multiple contacts' since the latter does not differentiate between the binding of a protein to separate alkyl residues or to different segments of one and the same alkyl residue. At high salt concentrations, protein binding displays a positive temperature coefficient in agreement with hydrophobic interactions (see **Figure 2**). A mathematical model of cooperative protein binding to an immobilized alkyl residue lattice was also developed allowing an estimation of the minimum number of alkyl residues (see **Figure 2B**) interacting with the protein. The model of multivalence was confirmed by equilibrium binding studies of phosphorylase *b* with alkylamines at high salt concentrations.

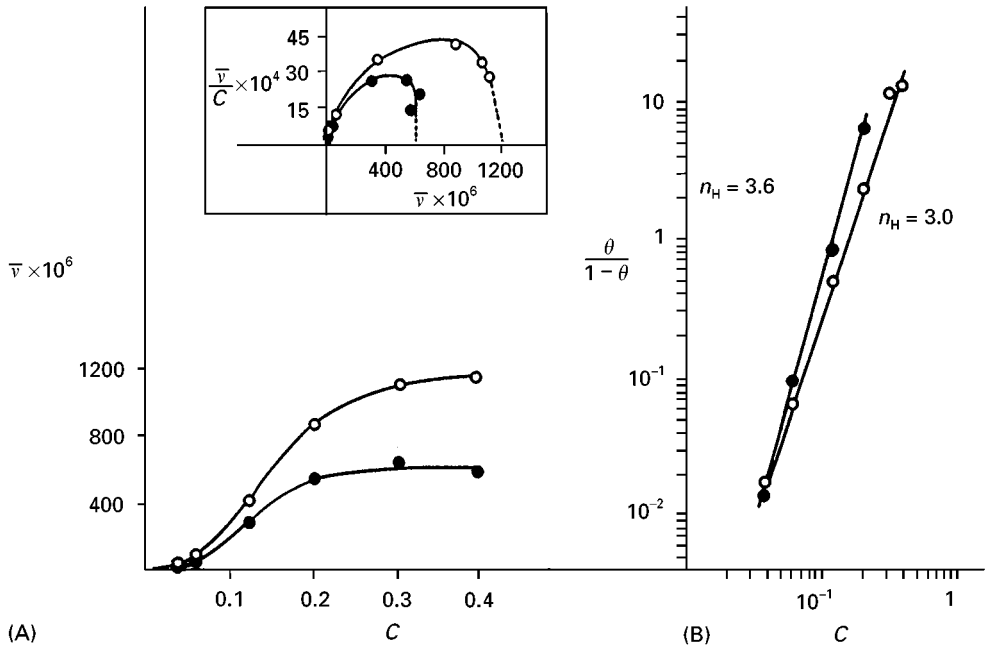

Figure 2 Dependence of the adsorption of phosphorylase *b* on the surface concentration parameter of Seph-C_4 at 5°C and 34°C at high ionic strength. The adsorbed amount of phosphorylase in the presence of 1.1 M ammonium sulfate was calculated from adsorption isotherms measured at each point at an apparent equilibrium concentration of free bulk protein of 0.07 mg mL^{-1}. The adsorbed amount of enzyme ($\bar{v}$) is expressed in relation to the anhydrodisaccharide content of agarose in mol^{-1} anhydrodisaccharide. Similarly *C* indicates the immobilized butyl residue concentration in relation to the anhydrodisaccharide content of agarose in moles of alkyl residue per mole of anhydrodisaccharide. A monomer molecular mass of 10^5 was employed for phosphorylase *b*. The alkyl agaroses were synthesized by the CNBr method. (A) Adsorption isotherms ('lattice site binding function') of phosphorylase *b* in Cartesian coordinates. Inset: Scatchard plots of the sigmoidal binding curves with extrapolation of fractional saturation of 610 (5°C) and 1220 (34°C) μmoles enzyme per mole of anhydrodisaccharide (corresponding to 6.2 and 13.4 mg mL^{-1} packed gel respectively). The broken lines indicate the mode of extrapolation. (●) 5°C; (○) 34°C. (B) Hill plots of the sigmoidal binding curves. θ the fractional saturation was calculated from the extrapolated saturation values of the Scatchard plot (A). The Hill coefficients n_H are given in the graph. The apparent dissociation constants of half-maximal saturation ($K'_{D,0.5}$) are 0.137 and 0.167 mole butyl residue per mole of anhydrodisaccharide at 5°C and 34°C respectively (which corresponds to 14.0 and 17.0 μmole butyl residues per ml packed gel, respectively). (●) 5°C; (○) 34°C. For further details see the text and for the source see Jennissen HP (2000) Hydrophobic (interaction) chromatography. In: Vijayalakshmi MA (ed.). *Theory and Practice of Biochromatography*. Amsterdam: Harwood Academic Publishers.

Adsorption hysteresis An important consequence of cooperative multivalent protein binding on alkyl-substituted surfaces is protein adsorption hysteresis. Protein adsorption hysteresis implies that the adsorption isotherm is not retraced by the desorption isotherm, due to an increase in binding affinity after the protein is adsorbed. The binding affinity increase can be attributed to an increase in the number of interactions (multivalence) which can either be due to a reorientation of the protein on the surface or to a conformational change in which buried hydrophobic contact sites (valences) are exposed due to the surface binding strain on the adsorbed protein. Adsorption hysteresis provides evidence for the concept that protein adsorption to multivalent surfaces in general is thermodynamically irreversible ($\Delta_i S > 0$) and that a true equilibrium has not been reached. Another conclusion from this concept is that protein adsorption in hysteretic systems is, moreover, not thermodynamically but kinetically controlled. Thus adsorption hysteresis has a strong influence on hydrophobic interaction chromatography by leading to nonlinearity and skewed elution peaks in zonal chromatography and to 'irreversibility' in adsorption chromatography. Hysteresis can, however, be easily reduced by decreasing the surface concentration of immobilized alkyl residues.

The Salt Parameter

Salting-out and salting-in on hydrophobically substituted hydrophilic gels The enhancement of hydrophobic interactions by high salt concentrations was first shown by Porath on uncharged benzyl ether agarose and termed a 'salting-out phenomenon'. Trypsin inhibitor could be purified 25-fold after being adsorbed at 3 M NaCl followed by elution in buffer without salt. Proof as to the mechanism and principle underlying these salt effects came in simultaneous, independent reports that the effect of

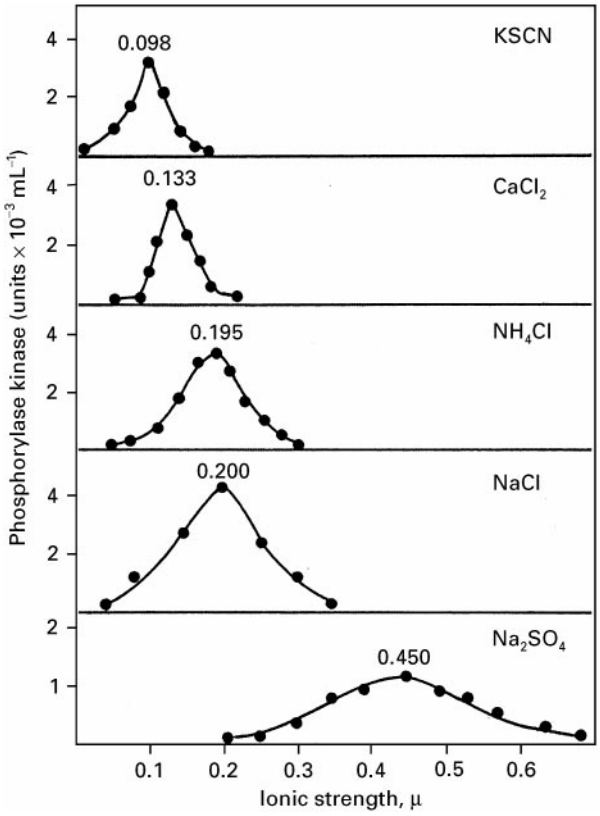

Figure 3 Influence of the salt parameter on the desorption (salting-in) of purified phosphorylase kinase from Seph-C_2 (25 μmol mL^{-1} packed gel) with salt gradients of different ionic composition. Each column with 5 mL of the above gel (CNBr method) was loaded with ca. 11 mg of the enzyme. The gradients were produced from 100 mL low ionic strength adsorption buffer and 100 mL salt containing buffer. The number at the maximum of the elution profiles indicates the ionic strength of the peak fraction. For further details see the text and for the source see Jennissen HP and Heilmeyer Jr LMG (1975) General aspects of hydrophobic chromatography. Adsorption and elution characteristics of some skeletal muscle enzymes. *Biochemistry* 14: 754–760.

salts on the adsorption and elution of proteins on alkyl agaroses indeed followed the Hofmeister series of salts. It could be shown that phosphorylase kinase was eluted ('salted-in') from a Seph-C_2-column by increasing salt gradients. The ionic strength of the peak fractions eluted, was inversely related to the salting-in power of the anions in the gradient in agreement with the Hofmeister series of salts (see **Figure 3**). Similarly proteins could also be eluted (salted-in) from uncharged octyl-Sepharose by an increasing salt gradient of $MgCl_2$ as shown by Raymond. The opposite effect, namely, the salting-out of phosphorylase *b* by ammonium sulfate on a Seph-C_1-column is shown in **Figure 4B**. The salted-out, i.e. adsorbed enzyme is eluted by omission of this salt from the buffer. Finally Pahlman showed that the salting-out power of anions, for the adsorption of human serum albumin (HSA) on uncharged Seph-C_5, also followed the order of the Hofmeister series of salts. All of these experiments clearly indicate that the action of the ions was not due to an electrostatic but to a lyotropic effect.

Theories of salt effects One of the earliest approaches to a theory of salt effects was based on the action of chaotropic ions on the solubility of proteins. The general conclusion of this work, with consideration of electrostatic and dispersion forces, was that chaotropes interacted indirectly with solutes (e.g. proteins) mainly through their effect on

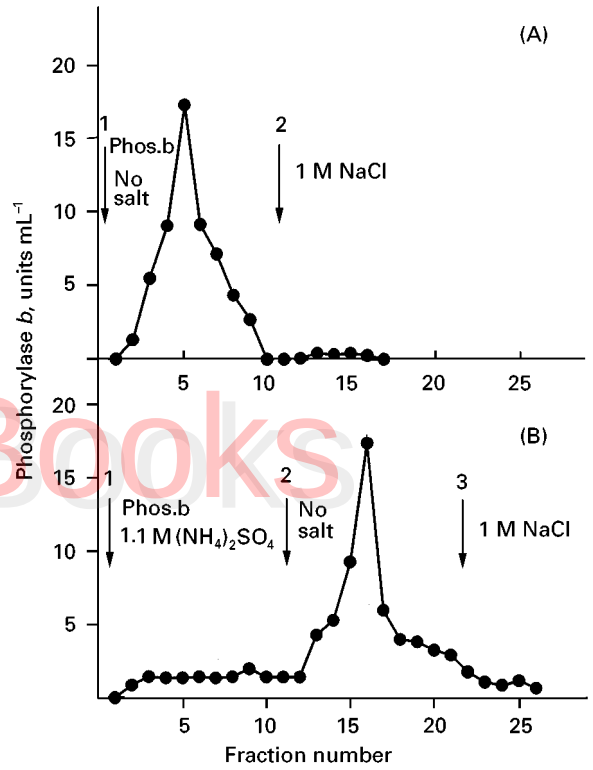

Figure 4 Influence of the salt parameter on the adsorption (salting-out) of purified phosphorylase *b* on Seph-C_1 (30 μmol mL^{-1} packed gel). The equilibration buffer contained 10 mM sodium β-glycerophosphate, 20 mM mercaptoethanol, 2 mM EDTA, 20% sucrose, 0.5 μM PMSF, pH 7.0 (buffer B) to which either 1.1 M ammonium sulfate or NaCl was added. 6 mg per 3 mL phosphorylase *b* was added to 20 mL Seph-C_1 in a 2 cm i.d. × 17 cm column. Fractions of 6.5 mL were collected. The gel was prepared by the CNBr procedure. (A) Application of enzyme to a column equilibrated with buffer without $(NH_4)_2SO_4$ or NaCl: (1) application of phosphorylase *b* in buffer B; (2) elution with buffer B + 1 M NaCl. (B) Application of enzyme to a column equilibrated with buffer with $(NH_4)_2SO_4$: (1) application of phosphorylase *b* in buffer B + 1.1 M ammonium sulfate; (2) elution with buffer B; (3) elution with buffer B + NaCl. For further details see the text and for the source see Jennissen HP (2000) Hydrophobic (interaction) chromatography. In: Vijayalakshmi MA (ed.). *Theory and Practice of Biochromatography*. Amsterdam: Harwood Academic Publishers.

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water structure. In the solvophobic approach of Horváth the surface tension of water was also of central importance. The energy necessary for bringing a solute into solution was equated with the energy needed for forming a corresponding cavity in water against the surface tension with a reduction in free volume. The net free energy involved in the association of two molecules was thus related to a reduction of the cavity size. Salting-in and salting-out were explained on the basis of the respective surface tension-decreasing/-increasing effect of the salt which was applicable to reversed-phase chromatography as well as to hydrophobic interaction chromatography. Finally, according to Arakawa (1984, 1991) there also do appear to be specific salt effects resulting from a direct binding of salt ions (e.g. of $MgCl_2$) to the protein.

Irrespective of the mechanism, the applicability of the Hofmeister (lyotropic) series of salts expanded by the chaotropic series, to hydrophobic interaction chromatography has been verified by many groups and these salts are important tools in controlling the adsorption and elution of proteins on these resins. The individuality of each protein in its quantitative interactions in such a system especially when in the native state should, however, not be underestimated.

Optimization of Hydrophobic Chromatographic Systems

Twenty years after the introduction of hydrophobic interaction chromatography, the method has not gained the same foothold in the methodological repertoire of protein chemistry as has affinity chromatography. Although a large number of proteins have been successfully purified by this method a recent paper by Oscarsson *et al.* comes to the conclusion that certain 'classical' commercial hydrophobic adsorbents are inadequate for ideal downstream processing because of their high hydrophobicity. The criticism of these authors is essentially correct. The major problem encountered on such hydrophobic gels is that proteins can be very effectively adsorbed but elution in the native state is often impossible. Although a similar problem can be encountered in affinity chromatography, it appears to be the major handicap in hydrophobic interaction chromatography and must be taken into account in any general optimization procedure.

The Homologous Series Method

Shaltiel's homologous series method of synthesizing hydrocarbon-coated agaroses was supplemented by the so-called exploratory kit, for choosing the most appropriate column and for optimizing resolution. This analytical kit, which was commercially available for some years, contained a homologous series of small columns from Seph-C_1 to Seph-C_{10} with two control columns. The principle was to determine the lowest member of the homologous series capable of retaining the desired enzyme or protein. This column was then selected for the purification of the desired protein. In a second step it was attempted to increase resolution by optimizing the elution procedure which ranged from mild salting-in procedures to reversible denaturation steps. This procedure or variants thereof are still the method of choice for most groups. However as illustrated by Oscarsson *et al.*, the number of failures is probably very high.

The Critical-Hydrophobicity Method

As stated above there are two methods for the synthesis of controlled hydrophobicity gels (a) via the homologous series of hydrocarbon-coated Sepharoses (variation of alkyl chain length) or (b) via the concentration series (variation of the alkyl surface concentration). The importance of the latter series has been underestimated. Both gel series essentially correspond to members of 'hydrophobicity gradients'. Although the decisive importance of the immobilized alkyl residue concentration for the hydrophobic adsorption of proteins (critical hydrophobicity) has been stressed for many years, no hydrophobicity gradient gel series has ever been produced commercially. Against the background of obvious problems in hydrophobic interaction chromatography, a novel rational basis for the optimization and design of such chromatographic systems has been suggested.

High yields in hydrophobic interaction chromatography can only be obtained if the protein to be purified is fully excluded from the gel under elution conditions as near as possible to physiological conditions, i.e., at low ionic strength. This means that the gel should be fully non-adsorbing under these conditions. On the other hand, since a purification is only possible if the protein is adsorbed to the gel, the matrix should be constructed in a way that adsorption can be easily induced by other means without denaturing the protein. Thus working at, or near to the critical hydrophobicity point, could solve both problems. In the synthesis of such critical-hydrophobicity gels the charge-free immobilized residues should be restricted to alkane derivatives, to ensure a 'purity' of hydrophobic interactions. NaCl, centrally located in the Hofmeister series, appears to be ideal. The procedure involves three steps: (i) selection

of an appropriate alkyl chain length, (ii) determination of the critical surface concentration of alkyl residues (critical hydrophobicity), and (iii) determination of the minimal salt concentration (NaCl) necessary for the complete adsorption of the protein. The three parameters are determined by a form of quantitative hydrophobic interaction chromatography utilizing primarily the high-affinity adsorption sites.

Selection of the appropriate alkyl chain length In the first step, an experimental setup similar to the homologous series method of Shaltiel is employed to gain information on the general hydrophobic binding properties of the protein and columns. However it is essential that a quantification of the immobilized surface concentration has taken place at this stage. Gels of 20–25 μmol mL^{-1} packed gel appear optimal. In general a constant amount of protein (ca. 0.5 mg mL^{-1} packed gel, which can be 100% adsorbed on the column of highest hydrophobicity) is applied at low or physiological salt concentration to each column (1–2 mL packed gel). One then determines the gel in the homologous series which adsorbs ca. 50% of the applied protein. In the case of the example below, ca. 50% of the applied fibrinogen was adsorbed on an uncharged Seph-C_5 gel containing 22 μmol mL^{-1} packed gel.

Determination of the critical hydrophobicity As previously defined, the critical hydrophobicity is that degree of substitution at which adsorption of a protein begins. As shown in **Figure 5** a strongly sigmoidal adsorption curve of fibrinogen is obtained on the concentration series of uncharged Seph-C_5 gels at a physiological NaCl concentration. The aim is to get as close as possible to the critical hydrophobicity point with a minimum of adsorbed protein. Since there was no measurable adsorption of fibrinogen at 12 μmol mL^{-1} packed gel and only ca. 2% was adsorbed at 13.6 μmol mL^{-1} packed gel (critical hydrophobicity), the ideal juxtacritical hydrophobicity range for fibrinogen was taken as 12–14 μmol mL^{-1} packed gel.

Determination of the minimal salt concentration (NaCl) necessary for adsorption In experiments with NaCl concentrations between 0.5 and 5 M, it was found that all of the applied purified fibrinogen was adsorbed on Seph-C_5 of a residue surface concentration of 13.6 μmol mL^{-1} packed gel at a salt concentration of 1.5–1.6 M NaCl. The salt concentration necessary for half-maximal adsorption was ca. 0.75 M NaCl. Since no (i.e., 2%) fibrinogen was

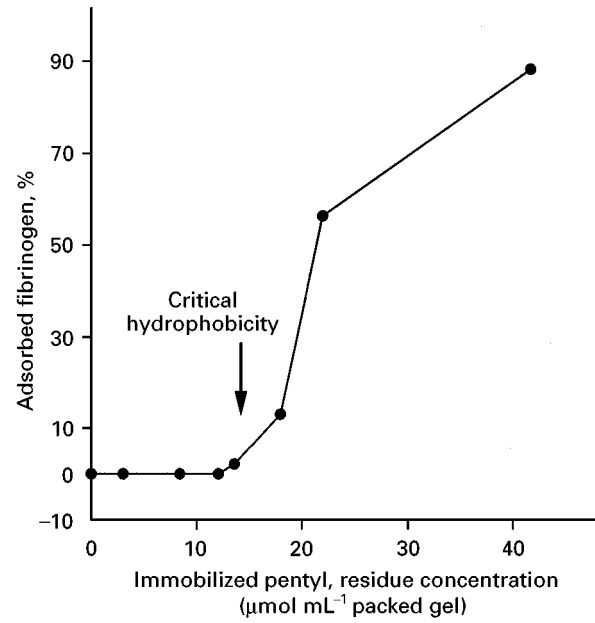

Figure 5 Determination of the critical surface concentration (critical hydrophobicity) of Seph-C_5 for the adsorption of purified fibrinogen. The uncharged pentyl agaroses were synthesized by the carbonyldiimidazole method. Purified human fibrinogen 1 mg) was applied in 1 mL to a column (0.9 cm i.d. × 12 cm) containing 2 mL packed gel in 50 mM Tris/HCl, 150 mM NaCl, 1 mM EGTA, pH 7.4. Fractions of 1.5 mL were collected. The column was washed with 15 mL buffer followed by elution either with 7.5 M urea or, at high hydrophobicity of the gel, with 1% SDS for the determination of the amount of protein bound. 100% equals 1 mg fibrinogen adsorbed to 2 mL packed gel of Seph-C_5. The total amount of adsorbed fibrinogen, corrected for the amount adsorbed to unsubstituted control Sepharose 4B, is shown. For further details see the text and for the source see Jennissen HP (2000) Hydrophobic (interaction) chromatography. In: Vijayalakshmi MA (ed.). *Theory and Practice of Biochromatography*. Amsterdam: Harwood Academic Publishers.

adsorbed to this pentyl Sepharose at low ionic strength, a complete recovery of fibrinogen adsorbed under these conditions is now possible by decreasing the salt concentration. Thus the critical hydrophobicity gel together with NaCl constitutes a fully reversible hydrophobic adsorption system for fibrinogen.

One-step purification of native fibrinogen from human blood plasma Employing Seph-C_5 of critical hydrophobicity equilibrated with 1.5 M NaCl it is possible to purify fibrinogen from human plasma in a single step (**Figure 6**). The procedure is so robust that fibrinogen can be purified from human blood plasma directly (no dialysis) in spite of a temporary decrease in NaCl concentration (fractions 5–9) during the run. After extensive washing with 1.5 M NaCl ca. 20-fold purified pure fibrinogen

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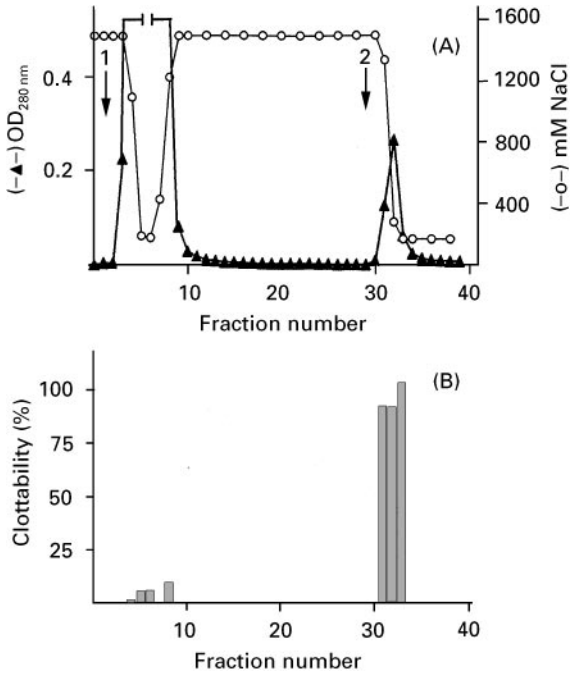

Figure 6 One-step purification of fibrinogen from human blood plasma by hydrophobic interaction chromatography at the critical hydrophobicity point of Seph-C_5. (A) 19 mL fresh unclotted human blood plasma was applied (arrow 1) to 20 mL packed Seph-C_5 (13.6 μmol mL^{-1} packed gel in a column 1.4 cm i.d. × 13 cm) equilibrated with 50 mM Tris/HCl, 1.5 M NaCl, pH 7.4 at a flow rate of 70 mL h^{-1} and a fraction volume of 6 mL. The nonadsorbed protein was washed out with 200 mL equilibration buffer. Elution (arrow 2) was facilitated by equilibration buffer containing a tenfold lower salt concentration of 150 mM NaCl. (B) The fractions 30–32 contain pure fibrinogen with a clottability of 93–100% with a total yield of 25%. For further details see the text and legend to Figure 5 and for the source see Jennissen HP (2000) Hydrophobic (interaction) chromatography. In: Vijayalakshmi MA (ed.). *Theory and Practice of Biochromatography*. Amsterdam: Harwood Academic Publishers.

(clottability 93–99%; **Figure 5**) is eluted by a negative step gradient from 1.5 to 0.15 M NaCl. The total yield is 25% with some loss in the run-through. Yields of fibrinogen of up to 60% have been obtained. If blood plasma equilibrated with 1.5 M NaCl is applied to the gel and eluted by a negative salt gradient, a clottability of 80% is obtained (**Figure 6**).

Conclusions

From the foregoing it can be concluded that hydrophobic interaction chromatography is one of the very basic separation methods in classical biochemistry. A great deal of information on the mechanisms involved in the method has been obtained and it appears that the critical hydrophobicity method for the optimization of hydrophobic supports offers a rational approach to the purification of proteins. The only drawback is that such hydrophobic gel series are not commercially available so that the application of this method necessitates experience in the synthesis of alkyl agaroses and the quantification of immobilized residues. Methodological investments of this types thus pose the 'high-energy barrier' to a more widespread and successful application of hydrophobic interaction chromatography in enzymology and protein chemistry.

See also: **I/Affinity Separation. III/Affinity Separation:** Liquid Chromatography. **Glycoproteins:** Liquid Chromatography. **pH-Zone Refining Countercurrent Chromatography:** High Speed Countercurrent Chromatography. **Polymers:** Field Flow Fractionation. **Appendix 1/Essential Guides for Isolation/Purification of Enzymes and Proteins.**

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